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Aberrations affecting the tumor suppressor gene *p16INK4a* have been described for a variety of tumors. In breast cancer, approximately 50% of tumors show low or lack *p16* expression. While evidence provided by some studies has implicated a possible role for *p16* in normal replicative senescence, other studies have suggested that the Rb pathway through which p16 functions may not be involved in senescence control. Previously we observed that all immortal lines derived from normal mammary epithelium which were analyzed for *p16* displayed inactivation of this gene through distinct mechanisms, supporting *p16* inactivation as a possible necessary event in escape from senescence. To further clarify this issue, we have analyzed p16 expression in a panel of normal finite lifespan human mammary epithelial cells (HMEC) from initial propagation through growth arrest, using media which confer different replicative capacity. Approximately 10-25 fold increase in *p16* expression was observed for all normal HMEC with initial onset of a senescence phenotype following 15-25 population doublings in culture. These cells also displayed expression of the senescence associated β-galactosidase. Interestingly, HMEC with additional long term replicative capacity arose from these growth arrested cultures, showing lack of p16 expression. This extended growth capacity appears to be associated with a methylation phenomenon since treatment of these cells with a methylation inhibitor resulted in growth arrest concurrent with reacquisition of p16 expression and senescence associated b-galactosidase. These results support *p16INK4a* as the 9p senescence gene and suggest a role for *p16* loss in the escape from initial onset of senescence and in acquisition of an extended life span of human mammary epithelial cells.

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The p16 Pathway in Breast Cancer and Senescence Control

INTRODUCTION

The $p16^{INK4a}$ protein, hereafter referred to as p16, is a known negative regulator of cell cycle progression through its cyclin dependent kinase (CDK) inhibiting function (1). p16 competes with the activating D type cyclins for association with CDK4 or CDK6, thereby preventing phosphorylation of proteins controlling G1 exit such as the retinoblastoma (Rb) protein (2). Inactivation of *p16* has been observed in numerous tumor types (3-8), and lack or reduced expression of *p16* has also been shown through a variety of technical approaches to occur in at least 50% of the breast cancer samples examined (9, 10). While these findings suggest that *p16* may play an important role in breast tumorigenesis, the consequence of such abberrations of *p16* are not yet clear.

Of the putative functions of *p16*, the role as a candidate senescence gene is supported by several observations. The mapping of *p16* to chromosomal subregion 9p21, a region containing a putative senescence gene, is significant in this regard (11). Also, p16 is an upstream negative regulator of retinoblastoma protein phosphorylation (1). Studies by Stein et al. have shown that replicative fibroblasts phosphorylate Rb upon stimulation with serum following serum starvation, but in senescent fibroblasts, Rb remains unphosphorylated (12). Analogously, elevated p16 levels are seen in senescent fibroblasts as compared to young fibroblasts, suggesting that Rb remains unphosphorylated in senescent cells due to high levels of p16 (11, 13). These and other observations suggest *p16* may be important in the control of replicative senescence.

Although these observations support a putative role for *p16* in the senescence control of fibroblasts, the situation in human mammary epithelial cells has remained less clear. The human papilloma virus 16 (HPV) proteins E6 and E7, which interact with p53 and Rb respectively, are both required for the HPV mediated escape from senescence in fibroblasts leading to immortalization (14) . However, in human mammary epithelial cells, the presence of E6 alone has been found to be sufficient for escape from senescence and the induction of an extended replicative capacity leading to immortalization (14, 15). Further, mammary epithelial cells from patients with Li-Fraumeni Syndrome (germline mutation of p53) spontaneously immortalize *in vitro,* while stromal cells from these patients do not (16). Normal human mammary epithelial cells have also been shown to undergo immortalization when transfected with only a mutant *p53* (17). This has led to the suggestion that, unlike in fibroblasts, the Rb pathway through which p16 exerts its growth suppressive effect is not involved in senescence control of mammary epithelial cells (14, 18).

However, in more recent studies, it has been suggested that distinct susceptibility of human mammary epithelial cells to E6/E7 induced escape from senescence may be dependent upon the stage of culture (19). While escape from senescence has been achieved by HPV 16 E6 alone, this was only seen in cultures with long term growth potential which had emerged from a period of growth arrest termed "selection". In contrast, most early passage cells which had not yet entered selection were immortalized by E7 alone and not E6 (19). The difference between these preselection and postselection cell populations which results in distinct susceptibility to viral immortalization has not been clear. Further, the reasons cells arrest growth at this stage, as well as the means of spontaneous escape, have not been elucidated.

We previously observed the inactivation of *p16* in immortal breast epithelial lines derived from normal mammary epithelium through three distinct mechanisms; homozygous deletion (MCF10 and MCF12 cell lines) nonsense mutation with hemizygous loss (184 A1 cell line), or hypermethylation (184 B5 cell line) (20) and unpublished data). These findings supported p16 inactivation as a possible necessary event for escaping senescence. In this report, we sought to further characterize the role of *p16* in senescence control of human mammary cells.. Normal primary human mammary epithelial cultures (HMEC) were followed from initial propagation *in vitro* until growth arrest and subsequent extended replicative growth. Expression analysis of *p16*, as well as another candidate senescence control gene $p21^{WAF1}$, was performed at various time points. The HMEC were also monitored for replicative capacity and expression of the senescence associated β-galactosidase. These results have been reported in a recent publication Brenner et al. (Oncogene 17: 199-205, 1998).

MATERIAL AND METHODS

Cell Culture. Normal human breast tissue specimens from reduction mammoplasties were obtained and epithelial organoids were separated from stromal components as previously described (21). Organoids were cultured in parallel in either a complex serum containing medium, MM (22, 23), containing 0.5% fetal bovine serum, conditioned media from the cell line Hs767Bl (30%), insulin (10 ug/ml), hydrocortisone (0.1 ug/ml), epidermal growth factor (5 ng/ml), and cholera toxin (1 ng/ml), or in the serum free medium MCDB 170 (23,24) supplemented with insulin (5 ug/ml), hydrocortisone (0.14 uM), epidermal growth factor (10 ng/ml), transferrin (5 ug/ml), isoproterenol (1 uM), and bovine pituitary extract (70 ug/ml)

For analysis of methylation, 5-aza-2'-deoxycytidine was added to a final concentration of 3.3 uM for 72-96 hours.

DNA and RNA Extraction. Total genomic DNA was isolated using phenol:chloroform:isoamyl alcohol (25:24:1) in Phase Lock Gel tubes (5 Prime→3 Prime, Boulder, CO), according to standard protocol, and precipitated with 2.5 volumes ethanol. Total RNA was isolated using an RNEasy Total RNA kit (Qiagen, Chatsworth, CA) as per manufacturers instruction.

Southern Analysis for Hypermethylation. Methylation analysis was performed as previously described (9). Briefly, 10μg of genomic DNA were digested with a flanking site enzyme (either Eco RI or Hind III) and a methylation sensitive endonuclease (Sac II, Sma I, and Eag I). The digested fragments were ethanol precipitated, resuspended, and resolved in 1% agarose gel overnight. DNA was transferred to a Zeta-Probe nylon membrane (BioRad, Richmond, CA) and hybridized with a 340 bp or 280 bp α-32P dCTP random prime labeled PCR fragment including exon 1α, as described (3). Autoradiographs were obtained following 2-4 days of exposure.

Duplex RT-PCR. Five μg of total RNA were used for first strand cDNA synthesis with Superscript II reverse transcriptase (Gibco BRL, Gaithersburg, MD) as per manufacturers instructions. Following reverse transcription, 4 μl (1/15 of total) each sample was subjected to amplification with 200 μM each primer, 2.0 mM $MgCl_2$, 1X Taq Buffer, 10% dimethylsulfoxide, 0.7 mM dNTPs, in a final volume of 20 μl. Following a 5 minute denaturing at 96°C, samples were held at 90°C while 5 units of Taq DNA Polymerase (Promega) were added, followed by twenty cycles at 94°C, 50°C, and 72°C for 40 seconds each. Reaction products were diluted fifty fold, and subjected to an additional 13 cycles (*p16*) or 10 cycles (p21) of amplification utilizing one nested ^{32}P polynucleotide kinase end labelled primer per reaction product, employing the same conditions as the first amplification. Reaction products were resolved on 5% (1:30) native polyacrylamide, followed by autoradiography. Signal intensities in all cases were analyzed and quantified with a Molecular Dynamics Phosphorimager.

Primers used were as follows: GAPDH 5', CCGATGGCAAATTCGAT GGC; GAPDH 3', GATGACCCTTTTGGCTCCCC; P16 5', CAACGCACCGA ATAGTTACG; P16 3'(1), AGCACCACCAGCGTGTC; P16 3'(2), CGTGTCCA GGAAGCCC; GAPDH 3'(P21), CAGAGATGATGACCCTTTTGGC; P21 5'(1), CAGCAGGAAGACCATGGTG; P21 5'(2), GACCTGTCACTGTCTTGTACC; P21 3', CCTGTGGGCGGATTAGGGCTTCC.

SA B-Gal Activity Staining. Cells were washed 2X with PBS, fixed in neutral buffered formalin, and stained as previously decribed (24). Briefly, following fixation, cells were incubated overnight at 37°C in a reaction buffer containing X-gal (1mg/ml), 40mM citric acid/sodium phosphate (pH 6.0), potassium ferrocyanide/ferricyanide (5 mM), NaCl (150 mM), and 2 mM $MgCl_2$. Negative activity control was breast carcinoma line T47D and positive activity control were senescent diploid fibroblasts.

RESULTS

Analysis of *p16* in Normal HMEC. Normal HMEC *in vitro*, like normal fibroblasts, undergo a limited number of cell divisions (25). In the case of HMEC, the total number of population doublings which can be achieved, as well as other characteristics, has been shown to be dependent upon the type of media used to propagate the cells (25). Specifically, finite lifespan HMEC grown in the serum containing medium, MM, display active growth for 2-5 passages, or 15-25 population doublings, with

gradual loss of proliferative activity. The senescent population retains the typical epithelial morphology (23). In contrast, when HMEC are grown in the serum-free MCDB 170 medium, after active proliferation for 2-3 passages, almost all the cells cease growth, becoming large, flat, striated, with irregular cell borders (23, 26). Following 2-4 weeks of inactivity, the population then undergoes a process termed "self-selection" (26). There is active proliferation of small cells with the typical epithelial cobblestone morphology, which soon dominate the culture. These post selection cells maintain growth for an additional 7-24 passages (approximately 45-100 population doublings in total), after which flatter and more vacuolated cells appear which retain the epithelial morphology without further growth arrest.

To assess the possible role of p16 in HMEC senescence, HMEC from five different reduction mammoplasty specimens were cultured in both MM and MCDB 170 and followed from initial propagation through cessation of growth. Initially, the levels of *p16* were low in both culture conditions. These levels rose with increasing passage until initial growth arrest, when *p16* levels were 10-25 fold higher than original values (Figure 1A and C, Figure 2). In contrast, assay of the emergent post-selection cells in MCDB 170 indicated that the level of *p16* transcript was drastically reduced (Figure 1C and D; Figure 2). None of the post-selection HMEC revealed expression of *p16* during the following growth period, nor at the final growth arrest. These findings were confirmed at the protein level as well. Figure 3 shows an immunoblotting analysis of the 184 HMEC culture using an anti-p16 antibody. Cells grown in serum containing media (MM) start with low levels of p16 protein expression (Fig. 3 MM passage 2) which increases and remains high until final growth arrest (Fig. 3 MM passage 6). On the other hand and as observed with the p16 transcript analyses, when HMEC are grown in serum-free MCDB170 medium, p16 protein is detected in the pre-selection cells (Fig 3. 170, passage 2) but not in the post-selection cell population (Fig 3. 170, passage 20).

Methylation of Post-Selection HMEC. Previous analysis of tumor cell lines as well as numerous primary tumors has shown inactivation of *p16* through de novo methylation of the promoter region (5). Promoters silenced by methylation can be reactivated in many cases by treatment with 5-aza-2'-deoxycytidine, a drug which is an established inhibitor of DNA methylation. In order to determine whether cells arising after initial arrest in MCDB 170 lacked expression due to methylation, post selection cells were grown in the presence of 5-aza-2-deoxycytidine and evaluated for *p16* expression. Of the 5 post-selection cultures examined, all regained *p16* expression following treatment (Figure 1C; Samples labelled PA, Figure 2). Further, analysis of the 184 post-selection cells by methylation-sensitive endonuclease digestion of DNA followed by Southern hybridization with a *p16* exon 1 probe, showed patterns consistent with complete methylation of *p16*, corroborating the aforementioned results (not shown). These data indicate that those cells capable of post-selection growth were inactive in *p16* expression due to de novo methylation.

Immunoblot Analysis. Protein expression analysis was performed using an anti-p16 antibody as previously described (27).

Coincidence of Expression of p16 and Senescence Associated β-galactosidase. In analysis of senescent cultures, Dimri et al. (24) observed the expression of an unusual endogenous β-galactosidase with a pH optimum of 6.0 This form of β-galactosidase was expressed by senescent cells *in vivo* as well as those cultured *in vitro*, but not by presenescent, quiescent, or terminally differentiated cells. While the source or function of this novel β-galactosidase is unknown, it nevertheless constitutes a useful senescence associated marker. To establish whether this marker showed coincident expression with growth arrest and increased *p16* expression of the HMEC grown in both culture conditions, cells were evaluated at various time points for the presence of senescence-associated β-galactosidase (SA β-Gal).

As expected, analysis of SA β-Gal in the early actively growing HMEC in either MM or MCDB 170 revealed no activity. As previously mentioned, these same cell populations also showed low *p16* expression. However, when cells ceased replicating following the 2^{nd}-5^{th} passage in both media, high levels of SA β-Gal were displayed, coincident with the highest levels of *p16* (Figure 4A and B). In contrast, the newly emergent post-selection HMEC from the same cultures in MCDB 170, which had no *p16* expression due to apparent hypermethylation of the *p16* promoter, did not display SA β-Gal activity positive cells. When these actively growing cells were treated with 5-aza-2-deoxycytidine to reverse methylation, cells ceased growing, regained expression of *p16* (as mentioned above), and expression of SA β-Gal was again observed concurrently (Figure 4E).

Analysis of p21^{WAF1} Expression with Senescence. Another CDK inhibitor, *p21^{WAF1}*, has been shown to be induced during senescence in fibroblasts (13, 28). In order to determine whether *p21* expression is associated with senescence of HMEC, levels of *p21* expression were followed in the aforementioned cultures. High levels of *p21* were observed during initial propagation, and showed little variability during growth arrest or extended replicative life (Figure 5). Thus, while increased expression of *p21* was seen with senescence in some cell types, this does not appear to be the case with HMEC, suggesting that *p21* does not play as significant a role in senescence of HMEC as *p16^{INK4a}*. This is similar to observations in adrenocortical cells, which express high levels of *p21^{WAF1}* throughout their replicative life span to senescence (29).

DISCUSSION

As previously mentioned, the number of population doublings which can be achieved in the culture of HMEC has been shown to be largely dependent upon the type of culture media used (25). Nevertheless, all HMEC undergo a period of initial growth arrest at an approximately similar level of growth, from which only those cells grown in serum free media recover. The reason cells undergo this initial growth arrest and the mechanisms by which cells recover from it, have not been understood. In the series of HMEC examined in this report, all showed an increase in *p16* expression with progression toward initial growth arrest, at which point the highest levels of *p16* were observed. When grown in serum free media, foci of actively growing cells emerged from these seemingly senescent cultures at a low frequency (~10^{-5}), and were devoid of *p16* expression due to possible hypermethylation of the *p16* promoter region. These cells did not regain *p16* expression at anytime during their remaining replicative period, nor at final growth arrest (Figure 3). These data would suggest that increased expression of p16 may be causative of the inital growth arrest observed in these cultures.

It has been suggested (30) that initial growth arrest of HMEC may be indicative of a normal senescence and an early mortality stage which is distinct from of those previously postulated in the Shay et al. (18) two stage model of human mammary epithelial immortalization. Indeed, the increased expression of *p16* in fibroblasts with onset of senescence has been previously described (13), and we now show a similar increase with the onset of initial growth arrest in HMEC. Additionally, our finding of expression of senescence marker SA β-Gal in cells at this initial growth arrest, as well as in those post selection cells growth arrested with regain of p16 expression when treated with a methylation inhibitor, adds weight to this prospect. Nevertheless, while SA β-Gal may be the best marker available for determination of senescence, it is not an absolute indicator. Further, SA β-Gal was also observed during the final growth arrest which has classicaly been viewed as senescence. Additional studies are needed to better address how the initial arrest and final arrest may relate to senescence *in vivo*.

As previously mentioned, analysis of immortalization of human mammary epithelial cells by E6 and E7 genes has shown susceptibilities distinct to those found in fibroblasts (14). Therefore, it has been proposed that while both Rb and p53 are involved in fibroblast immortalization, only p53 appeared to play a role in mammary epithelial cells (14, 18). However, it is worth noting that the analysis of susceptibility to immortilization by E6 and E7 was performed with "post-selection" mammary epithelial cells grown in media MCDB 170, which we have now shown to lack *p16* expression (14). This would suggest that the lack of necessity for E7 in immortalization of mammary epithelial cultures is due to prior inactivation of *p16*, and subsequent alteration of the Rb pathway. In such circumstances, the sequestration of Rb protein by E7 may confer no known additional benefit. Further, it suggests that similar to fibroblasts, both Rb and p53 dependent pathways play a role in the senescence control of mammary epithelial cells.

In support of this argument, recent studies by Wazer et al. (19) have revealed that mammary epithelial cells present in milk and grown in a high serum supplemented media, similar to cells grown in serum supplemented media MM which we show to maintain *p16* expression, show substantial extension of life span with E7. While E6 was not able to confer a similar extension of life span in these cultures, its presence was required for complete immortalization. Additionally, in similar analysis of susceptibility to E6 and E7 immortalization of pre-selection and post-selection mammary epithelial cultures from tissue grown in media DFCI-1, a medium of constitution similar to MCDB 170 which results in selection, Wazer et al. (19) were able to demonstrate that most pre-selection HMEC cultures undergo extension of lifespan followed by immortalization with E7 and not E6. In contrast, post-selection HMEC immortalize with E6

and not E7. This was subsequently corroborated by others, and the arrest which E7 is capable of circumventing was designated M0 by Foster and Galloway (30) to conform to nomenclature previously used in a two stage model of immortalization suggested by Shay et al. (14). Thus, the requirement for E7 in immortalization of cells expressing *p16* (pre-selection), and lack of necessity for E7 in the immortalization of mammary epithelial cells with inactivated *p16* (post-selection), supports the role of *p16* as a senescence control gene in human mammary epithelial cells. Additionally, this suggests that a chronology exists in the senescence control of human mammary epithelial cells where increased p16 expression may be invoved in an initial stage (M0) of normal growth arrest. Inactivation of this initial control stage (e.g. p16 inactivation or Rb sequestration by E7) appears to result in a limited increase in replicative capacity and suspectibility to further extension of lifespan by circumvention of the second (M1) stage (e.g. by E6 or mutant p53 (14). Following a final period of selection (crisis, M2) immortal cells emerge. This may be typified by previous experiments such as those of spontaneous immortalization of post-selection (grown in MCDB 170) mammary epithelial cells from Li-Fraumeni patients, while fibroblasts from these same patients required E7 to immortalize (16). Adaptation of previously suggested two stage models of mammary epithelial cell immortalization to include a first step dependent upon Rb pathway inactivation might be necessary.

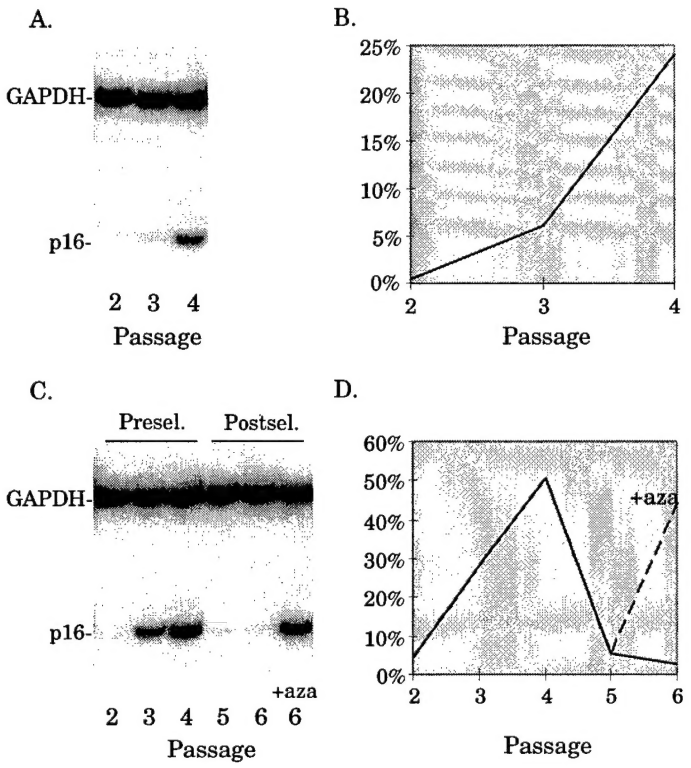

Figure 1. Expression of p16 in normal human mammary epithelial culture B26 from early culture through selection. **A, B.** Duplex RT-PCR analysis of B26 grown in media MM reveals an increase in p16 expression relative to glyceraldehyde 6-phosphate dehydrogenase (GAPDH) expression with increased passage. Highest expression at P4 coincided with growth arrest. **C, D.** Analysis of B26 grown in media MCDB 170 reveals a similar increase in relative expression. However, following growth arrest at P4, small cells emerge which apparently lack expression and soon dominate the culture. When treated with 3 μM 5-aza-deoxyctidine (aza) for 72 hours to inhibit methylation, cells arrest growth and p16 levels rebound to those observed at P4.

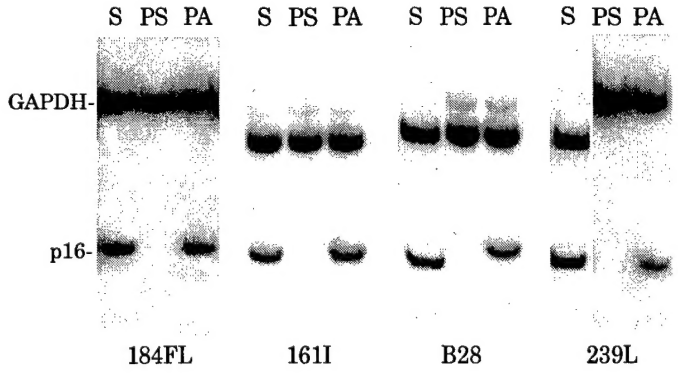

Figure 2. Duplex RT-PCR analysis of p16 expression relative to glyceraldehyde 6-phosphate dehydrogenase (GAPDH) expression in four additional normal human mammary epithelial cultures at initial growth arrest (S), post-growth arrest (PS), and when treated with a methylation inhibitor post-growth arrest (PA). Notice all cultures express high levels of p16 at initial growth arrest, but not following unless treated with a methylation inhibitor.

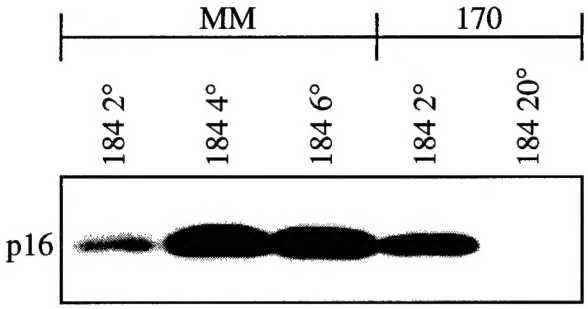

Figure 3. Immunoblot analysis of p16 protein expression in normal 184 human mammary epithelial cells. Early in culture, cells grown in serum containing media (MM) show low levels of p16 protein expression (MM passage 2) which increases in subsequent passages and remains high until final growth arrest (MM passage 6). The same HMEC grown in serum-free MCDB170 medium (170) show detectable p16 protein only in pre-selection cells (170, passage 2) but not in post-selection cells (170, passage 20). All lanes were control for equal protein loading (not shown).

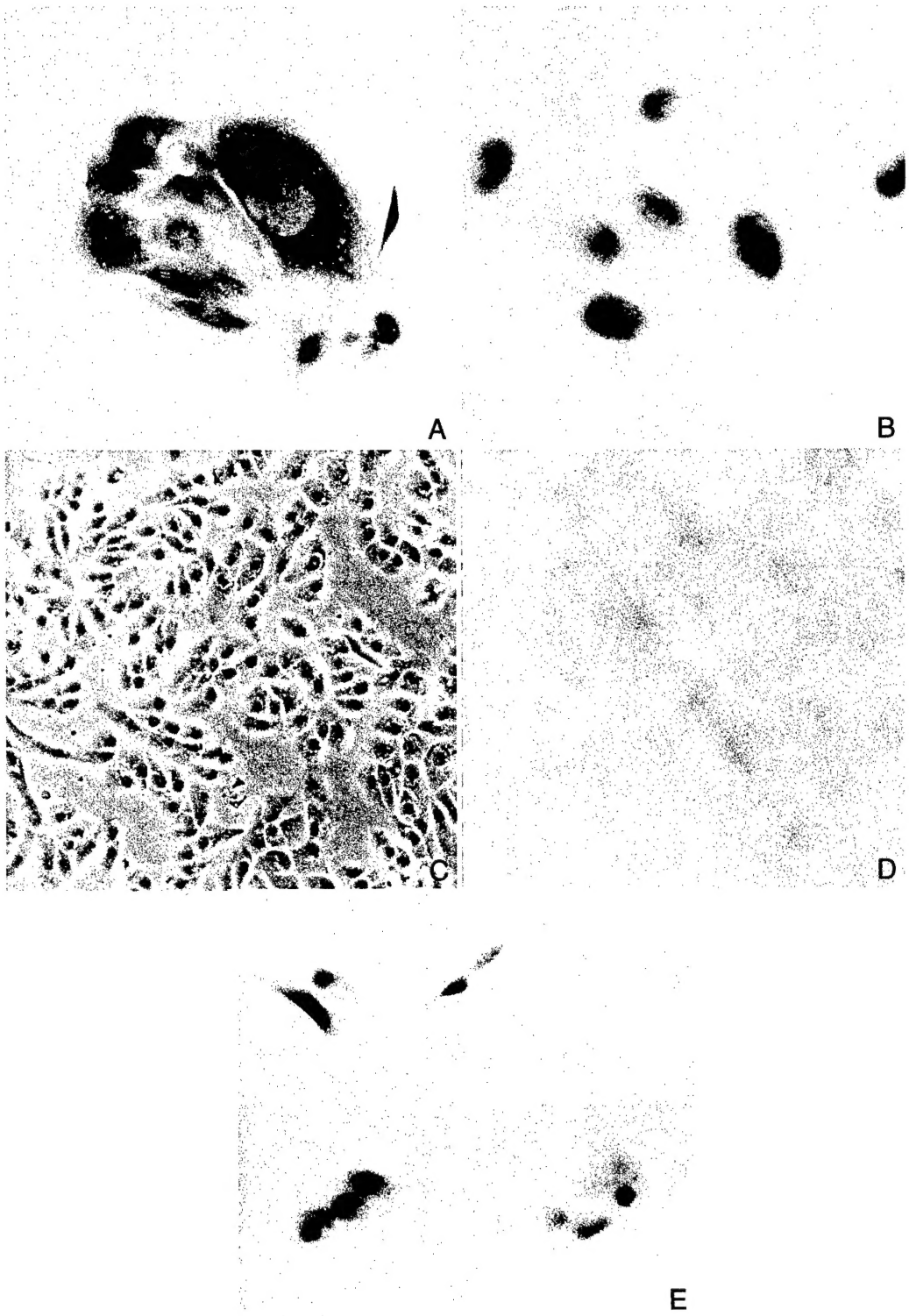

Figure 4. Analysis of senescent associated beta-galactosidase (β-gal)in normal human mammary epithelial cultures. **A.** Representative β-gal staining from a terminally arrested culture in media MM (bright phase). Culture shown is B26 at passage 4. **B.** Representative β-gal staining of a first arrest culture in media MCDB 170 (bright phase). **C.** Post-selection cells (phase contrast), culture 184. **D.** Same field as C (bright phase). **E.** Post selection cells treated with 3.3uM 5-aza-2-dexvcvtidine for 72 hours. culture 184.

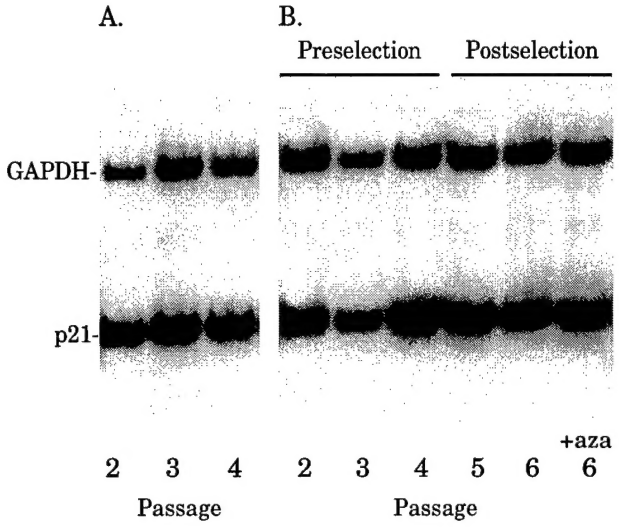

Figure 5. Expression of p21 in normal human mammary epithelial culture B26 from early culture through selection. **A.** Duplex RT-PCR analysis of B26 grown in media MM reveals little relative change in p21 expression relative to glyceraldehyde 6-phosphate dehydrogenase (GAPDH) expression with increased passage. **B.** Analysis of B26 grown in media MCDB 170 reveals a similar lack of relative change in expression, even when treated with 3.3 μM 5-aza-2-dexycytidine (aza) for 72 hours.

CONCLUSIONS

In this study we show an increase in expression of *p16* with HMEC growth *in vitro*, the highest levels of *p16* expression coinciding with the first growth arrest and expression of a senescence marker, SA β-Gal. Cells emerged from these seemingly senescent cultures devoid of *p16* expression possible due to hypermethylation of the *p16* promoter region. No change was seen in $p21^{WAF1}$ expression from initial propagation through the entire culture period. These results support *p16* as well as the Rb pathway in the senescence control of normal mammary epithelial cells. Further, these results provide for the first time insight into the reasons for the different behavior of stromal and epithelial cells observed in previous studies of induced extended life and immortalization

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